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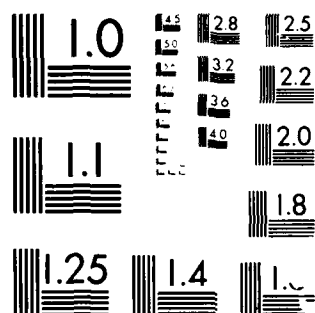
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# MECHANISM OF CHEMICAL ACTION AND TREATMENT OF CYANIDE POISONING

## ANNUAL PROGRESS REPORT

Charles A. Tyson, Ph.D.

OCTOBER 1, 1984

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<p>In the second year under this contract, the combined hepatocyte/erythrocyte <u>in vitro</u> system developed in the first year was applied to the study of cyanide and antidotal mechanisms of action. Sodium nitrite and 4-dimethylaminophenol were found to reverse cyanide depression of ATP levels in hepatocytes by catalyzing the formation of methemoglobin in erythrocytes coincubated with the hepatocytes. Cobalt(II) chloride and sodium thiosulfate operated by different mechanisms. Cobalt chloride reversed ATP depression presumably by complexing free cyanide ion in the reaction medium directly since neither cyanmethemoglobin nor thiocyanate was formed in appreciable quantities during the process. In the case of sodium thiosulfate, sulfane sulfur was transferred enzymatically to cyanide to form thiocyanate, a less toxic ion, in near stoichiometric quantities. Hepatocytes and/or albumin in the reaction medium facilitated this reaction, but erythrocytes were also found to contribute to the rate of cyanide conversion to thiocyanate.</p> <p style="text-align: right;">(continued on back)</p>					
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19. ABSTRACT (concluded)

The relative effectiveness (on the basis of concentration) of the antidotes tested in a therapeutic protocol (antidote added after cyanide) was estimated to be: 4-dimethylaminophenol > cobalt(II) chloride >> sodium thiosulfate  $\cong$  sodium nitrate. Cobalt(II) bis-pyridyl, cobalt(II) chloride, and hydroxycobalamin were also tested in a prophylactic protocol (antidote added before cyanide) and were found to be effective in preventing cyanide-induced depression of ATP.

Comparative studies on cyanide cytotoxicity were also conducted with hepatocytes isolated from a beagle and from human liver biopsy specimens. Initial results indicated no pronounced differences in the response compared with that of rat hepatocytes, but additional experiments are required for a more definite conclusion.

## SUMMARY

In the second year under this contract, the combined hepatocyte/erythrocyte in vitro system developed in the first year was applied to the study of cyanide and antidotal mechanisms of action. Sodium nitrite and 4-dimethylaminophenol were found to reverse cyanide-induced depression of ATP levels in hepatocytes by catalyzing the formation of methemoglobin in erythrocytes coincubated with the hepatocytes. Cyanide complexed with the methemoglobin to form cyanmethemoglobin, which was quantitated directly in the erythrocytes; significant quantities of thiocyanate were not formed. Lowering the hemoglobin content in the medium below that required to complex the cyanide did not result in reversal of ATP depression.

Cobalt(II) chloride and sodium thiosulfate operated by different mechanisms. Cobalt chloride reversed ATP depression presumably by complexing free cyanide ion in the reaction medium directly, since neither cyanmethemoglobin nor thiocyanate was formed in appreciable quantities during the process. In the case of sodium thiosulfate, sulfane sulfur was transferred enzymatically to cyanide to form thiocyanate, a less toxic ion, in near stoichiometric quantities. Hepatocytes were able to catalyze this reaction in the absence of erythrocytes or albumin in the medium. However, adding erythrocytes significantly enhanced the rate of reversal of ATP depression and of cyanide conversion to thiocyanate. The effect of erythrocytes was greater than that of albumin.

The relative effectiveness (on the basis of concentration) of the antidotes tested in a therapeutic protocol (antidote added after cyanide) was estimated to be: 4-dimethylaminophenol > cobalt(II) chloride >> sodium thiosulfate > sodium nitrite. Cobalt(II) bis-pyridyl, cobalt(II) chloride, and hydroxycobalamin were also tested in a prophylactic protocol (antidote added before cyanide) and were found to be effective in preventing cyanide-induced depression of ATP.

Comparative studies on cyanide cytotoxicity were also conducted with hepatocytes isolated from a beagle and from human liver biopsy specimens. Initial results indicated no pronounced differences in response compared with that of rat hepatocytes, but additional experiments are required for a more definitive conclusion.

## FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH) 78-23, Revised 1978].

For the protection of human subjects, the investigator has adhered to policies of applicable Federal law.

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## INTRODUCTION

### Statement of Problem

This contract is concerned with the development and use of in vitro systems for the study of cyanide and antidotal mechanisms at the cellular level and the preliminary screening of antidotes for potential effectiveness. During this reporting period, the following activities were performed: (1) Data on cyanide cytotoxicity to hepatocytes in the in vitro system were refined and expanded; (2) further studies were performed on the two cell (hepatocyte/erythrocyte) coincubation system for validating antidotal response and mechanisms of action; and (3) cyanide was tested in hepatocytes from other animal species as the initial step for ultimately comparing interspecies differences in response in vitro with relative in vivo potency.

### Background

Hydrogen cyanide is an extremely fast-acting blood-gas poison that rapidly permeates cell membranes and inhibits respiration by forming stable complexes with cytochrome oxidase, the terminal enzyme in the mitochondrial electron-transport chain. The histotoxic anoxia that results is thought to be responsible for the neurological, cardiovascular, and lethal effects observed with cyanide poisoning in humans (1-3). Although the target enzyme for cyanide has been identified, the events leading to loss of function and cell death have not been defined. Such information could provide the basis for an economical in vitro approach to evaluating the potential efficacy of antidotes for preventing and treating cyanide poisoning as well as provide insight on their reaction mechanisms. These mechanisms are complex, and it is difficult to obtain definitive information on the various possibilities from whole animal studies alone (4-6). Furthermore, experimental data on the response of the same cell type from different species to hydrogen cyanide and of potentially effective antidotes against cyanide action would amplify the data base supporting the choice of the most appropriate animal model for humans and assist in developing a therapeutic or preventive regimen for the optimal antidote or antidote combination for human exposures. It is presumed (although this must be experimentally tested) that the functional deficits produced by cyanide at the cellular level in different species will parallel differences in species sensitivity to the toxin (7).

To accomplish these ends, we proposed to develop an in vitro approach to the problem, working initially with a coincubation system comprised of hepatocytes and erythrocytes. The choice of hepatocytes as a target cell was dictated primarily by an interest in documenting a role for liver

enzymes in cyanide detoxication and in interspecies differences in liver capability to metabolize cyanide, which may influence antidote efficacy (6,8,9). Erythrocytes are required to provide hemoglobin for those antagonists that effect recovery through inducing methemoglobin to complex cyanide (4,10). The system also incorporates albumin (0.2%) in the medium, which level can be lowered or increased to evaluate a possible role for this protein in sulfur transfer to cyanide (11,12). Manipulation of the components in the system and determination of the disposition of cyanide can help to clarify the requirements for antagonism of cyanide-induced functional deficits and the mechanisms involved.

One important goal of this research was to validate the coincubation system for use with cyanide antidotes. For this purpose, antidotes with widely different mechanisms of action were chosen. These included  $\text{NaNO}_2$  and 4-dimethylaminophenol (DMAP), which induce methemoglobin that complexes cyanide in the bloodstream (4), cobaltous chloride, which forms stable complexes directly with cyanide and excretes it in urine (13-15), and sodium thiosulfate, which converts cyanide to the less toxic thiocyanate ion with the aid of sulfurtransferases in tissues, primarily rhodanese in liver (12). The body of evidence for these mechanisms is fairly substantial, but there are experimental discrepancies or gaps that have raised questions concerning the accuracy of some of them. In regard to  $\text{NaNO}_2$ , Holmes and Way (16) reported that pretreatment of mice with methylene blue, an inducer of methemoglobin reductase, failed to prevent  $\text{NaNO}_2$  from antagonizing cyanide-induced lethality. It was proposed that alternative mechanisms, possibly physiologic, might actually be responsible for the antidotal action (6). In the case of  $\text{CoCl}_2$ , evidence for its mechanism of action in animals rests primarily on dosing studies that show it can protect against six lethal doses of cyanide, the same stoichiometry as required for formation of the hexacyanocobalt anion (14); detailed studies have never been conducted with the antidote and cyanide in biological systems to confirm this and work out the details. In the case of thiosulfate, a concern voiced by several investigators is that rhodanese in the inner mitochondria of tissues that detoxify cyanide is not readily accessible to thiosulfate, which permeates biological membranes poorly (11,12,17). In the process therefore of validating the system for antidotal studies, an additional aim of the research was to seek evidence to help resolve some of these mechanistic questions.

In Annual Report #1 (18), we reported on our development of a coincubation system comprised of hepatocyte monolayer culture and erythrocytes in suspension and our preliminary evaluation of the system as a screen for cyanide antidotes. When KCN was added to the incubation medium, it selectively depressed hepatocyte ATP levels and inhibited urea synthesis. On prolonged incubation (120 to 240 min) with the cytotoxin, hepatocyte LDH was released to the medium, whereas erythrocyte lactate dehydrogenase (LDH) content was unchanged even at KCN concentrations as high as 2.5 mM. Of the various cytotoxicity indicators investigated--ATP, urea synthesis,  $\beta$ -hydroxybutyrate to acetoacetate ratio,  $\text{Ca}^{2+}$  flux, and LDH release--ATP was chosen as the most suitable parameter because its level was directly related to  $\text{O}_2$  consumption levels, effects on it by cyanide were

reversible, and it was not a rate measure, which requires time to ascertain.

Using this indicator, it was shown that after a short incubation with KCN the depression in ATP levels was reversible on exchange of the medium with fresh medium containing no KCN. Alternatively, an experiment was conducted in which  $\text{NaNO}_2$ ,  $\text{Na}_2\text{S}_2\text{O}_3$ , or both in combination were added directly to the medium 10 min after KCN. The antidotes and antidote combination reversed ATP depression in the hepatocytes without removing cyanide from the medium. At a lower erythrocyte content in the system, insufficient to complex the cyanide added if hemoglobin were stoichiometrically converted to methemoglobin, sodium thiosulfate was again effective but  $\text{NaNO}_2$  was not. These initial results indicated that the coincubation system was responsive in the manner expected and had the potential for application as an antidote screen and for mechanistic studies.

## METHODS

### Chemicals

Sodium thiosulfate was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ), and cobaltous chloride was obtained from Sigma Chemical Co. (St. Louis, MO). 4-Dimethylaminophenol (DMAP) was supplied by the Walter Reed Army Institute of Research (Bethesda, MD) and was >97% pure. Potassium cyanide and sodium nitrite were purchased from Mallinckrodt, Inc. (Paris, KY). Potassium [ $^{14}\text{C}$ ]cyanide (specific activity, 8 mCi/mole) was obtained from New England Nuclear (Boston, MA). All other chemicals and biochemicals were reagent grade.

### Cell Preparation

Hepatocytes were isolated from the following species in these studies: rat, dog, and human. Adult male Sprague-Dawley rats, weighing 320 to 390 g (Simonsen Laboratories, Gilroy, CA) were housed in plastic cages containing PWI bedding. They were allowed food and water ad libitum and were maintained on a 12 hr light/dark cycle. Animals were anesthetized with pentobarbital and, immediately after liver removal, were euthanized by perforation of the diaphragm. Hepatocytes were isolated by collagenase perfusion, and cultures were prepared in a hormone-supplemented Waymouth's 752/1 medium using collagen-coated petri dishes as described in Green et al. (19). After attachment and replacement of the medium with fresh culture medium, hepatocyte cultures were incubated at 37°C under an air:CO<sub>2</sub> (95:5) atmosphere for 20 hr, at which time they were used for experiments. Hepatocytes had an average viability at isolation of 82%, as determined by trypan blue exclusion, and a plating efficiency of 64% ( $1.6 \times 10^6$  cells attached/60-mm diameter dish).

For erythrocytes, whole blood was collected from rats in heparinized syringes by cardiac puncture at the time of sacrifice for preparing hepatocytes. The blood was centrifuged at 850 x g for 10 min. The plasma and buffy coat were discarded, and the red blood cells were resuspended in culture medium (1:4, v/v) and stored at 4°C overnight. Just before their use in coincubation experiments, the red blood cells were centrifuged again, the medium was discarded, and the red blood cells were resuspended in culture medium (equivalent to 0.4 ml whole blood, or ~ 1.6 g hemoglobin/dL).

Isolation of hepatocytes from other species was performed by biopsy perfusion with collagenase using the method described for rats with minor modifications (20). The dog used for hepatocyte isolation was a young-adult female beagle obtained from Marshall Research Animals (North Rose,

NY). It had been sprayed with synthetic pyrethroids in ethanol as part of an unrelated research project 4 months previously. Human liver specimens were obtained from donors of organs for transplantation (heart or heart/lung and kidney) at Stanford University Medical Center. Both donors were young-adult male Caucasians who died from head injuries. One was 20 years old and the other was 35. After removal of the organs to be transplanted, liver specimens were excised and placed immediately in ice-cold buffer [ $\text{Ca}^{++}$ -free, containing ethylene glycol bis(8-aminoethyl ether) N,N'-tetraacetic acid as described in Green et al. (21)] for transport to the laboratory. Because of the close proximity of our laboratory to the hospital, human liver was obtained within 20 min of the cessation of blood flow, and the perfusion was started about 15 min later.

The cell viability of dog hepatocytes was 94% and the plating efficiency was 60%. Cell viabilities for the human hepatocytes averaged 89%, and plating efficiency averaged 60%.

Erythrocytes from the beagle were prepared from blood collected in vacutainers containing citrate dextrose as anticoagulant just before sacrifice in the same manner as for rats. Human blood was obtained in EDTA-containing vacuoles from the Red Cross (Palo Alto, CA) on the morning of experiments; erythrocytes were prepared as above. Experiments with rat erythrocytes and the different anticoagulants indicated that there was no substantial difference in sulfurtransferase activity in the cells.

#### Culture Experiments

For the cytotoxicity experiments, ground-glass weighing bottles (50-ml capacity, Kimble) were inverted and used as reaction flasks. The culture dishes were placed in the cover, and the medium was removed from each dish by aspiration and replaced with 4 ml of fresh, room-temperature medium containing the requisite amount of KCN. In experiments where urea synthesis was assessed, substrates (10 mM  $\text{NH}_4\text{Cl}$  and 10 mM ornithine) in 0.10 ml of medium were added to the dishes. The weighing bottle bottoms were placed over the dishes, and the atmosphere in the flasks was replaced with air: $\text{CO}_2$  (95:5) by gentle gassing for 1 min through a gassing port cut in the top. The ports were immediately closed with rubber stoppers, and the flasks were incubated in a shaking water bath at 37°C and 60 oscillations/min for the scheduled incubation time. The flasks were then removed from the water bath, and the medium was aspirated with a Pasteur pipette and saved for analysis of biochemical components. Hepatocyte adenine nucleotides were analyzed as described below. Duplicate flasks were used for each time point. Control flasks, also in duplicate and handled in the same manner except for omission of KCN, were run concurrently.

For antidote studies in the coincubation system, culture dishes were placed in ground-glass weighing bottles as above. The reactions were started in timed sequence by replacing the culture medium (4.0 ml) with an equal volume of fresh medium containing KCN at the desired concentration and erythrocytes [ $\sim 1.6$  g hemoglobin/dL, sufficient to bind all cyanide at



1.0 mM if the hemoglobin were totally converted to methemoglobin (8)] through a port in the top of the flask. After a 1-min exchange of the air atmosphere with air:CO<sub>2</sub> (95:5), the port was stoppered and the flasks were incubated in a shaking water bath at 37°C and 60 oscillations/min. After 10 min of incubation, 0.10 ml of stock antidote solutions was introduced quickly through the port, and the port was immediately closed to initiate the recovery phase of the experiment. Control hepatocyte cultures were prepared in the same manner except for omission of KCN and were run concurrently. Duplicate flasks were used for each time point.

For experiments to determine methemoglobin formation, the experimental conditions were the same, unless otherwise indicated, except for the omission of KCN from the system.

### Analyses

ATP was analyzed as follows: Immediately upon aspiration of the culture medium, 0.50 ml of Releasing Agent (Diagnostic Services, San Diego, CA) was added to the dish to lyse the cells. The hepatocytes were scraped from the dish and suspended with a Pasteur pipette in the Releasing Agent. A small aliquot of the suspension was taken and diluted (1:30) in 0.05 M HEPES + 4.0 mM MgSO<sub>4</sub>, pH 7.7. ATP analysis was conducted immediately on this diluted sample using a Packard Picolite Luminometer (Downers Grove, IL) and an ATP-coupled luciferin-luciferase assay (Diagnostic Services, San Diego, CA). Results were converted to molar concentrations of ATP per culture, based on a standard curve using equine muscle ATP (Sigma Chemical Co., St. Louis, MO) over the concentration range of 10<sup>-8</sup> to 10<sup>-4</sup> M.

Urea accumulation in the culture dishes (urea synthesis) was determined as follows. Aliquots (0.50 ml each) of the culture medium were transferred by pipette into screw-cap test tubes containing 50 µl of 60% PCA and were stored at -20°C until analysis. At analysis, the samples were neutralized with 0.10 ml of a solution containing 0.5 M triethanolamine and 3 M K<sub>2</sub>CO<sub>3</sub> and centrifuged to remove precipitate. The supernatant was analyzed spectrophotometrically for urea by the method of Foster and Hochholzer (22). Results are presented as µmol of urea/ml of culture medium.

Analyses of LDH, aspartate aminotransferase (AST), and acid phosphatase were conducted using a semiautomated Gemeni Miniature Centrifugal Analyzer (ElectroNucleonics, Fairfield, NJ) and reconstituted Beckman reagents (Beckman Instruments, Carlsbad, CA). Raw data are expressed as U/L enzyme activity. Net percentage release is the percent of the initial intracellular hepatocyte enzyme that is released by KCN. This value is derived from the raw data by subtracting the quantity of enzyme activity released by control cells from activity released by treated cells and from the total initial hepatocyte enzyme activity after lysing the cells with Triton X-100 (23).

Lactate was quantitated spectrophotometrically on 0.40-ml aliquots of the medium after centrifugation to remove erythrocytes by measuring the conversion of  $\text{NAD}^+$  to NADH at pH 9.5 with lactate dehydrogenase (24). Pyruvate was determined in reverse manner on separate aliquots (1.5 ml) at pH 7.6 with NADH as substrate (25).

Reduced glutathione (GSH) was determined by the method of Hissin and Hilf (26) as follows. Cells were scraped from three plates and pooled in 2.0 ml of buffer (0.1 M sodium phosphate, 5.0 mM EDTA, pH 8.0). A 0.5-ml aliquot was removed for DNA analysis. The remainder was frozen in dry ice/acetone and stored at  $-70^\circ\text{C}$  until analysis. After thawing, 0.4 ml of 25% metaphosphoric acid was added to precipitate protein. The sample was homogenized and centrifuged at  $100,000 \times g$  for 30 min. After 1:100 dilution in the phosphate-EDTA buffer, 0.1 ml of *o*-phthalaldehyde (1 mg/ml in methanol) was added to 1.9 ml of the diluted sample and allowed to stand for 15 min. The fluorescence of the sample was determined in an Aminco-Bowman Spectrophotofluorometer (American Instrument Co., Silver Spring, MD) at an emission wavelength of 420 nm using an excitation wavelength of 350 nm against GSH standards in the 0.1 to 2.0  $\mu\text{g/ml}$  range. DNA was quantitated spectrophotometrically by the method of Richards (27). One million rat hepatocytes contain approximately 10.9  $\mu\text{g}$  DNA (21).

For lipid peroxidation, a 0.25-ml aliquot of scraped cells was placed into a screw-top test tube containing 0.5 ml of 15% TCA and the mixture was frozen at  $-20^\circ\text{C}$  until analysis. After thawing, 1.0 ml of 0.67% thiobarbituric acid (TBA) was added, the tube was recapped, and the contents were immediately heated at  $92^\circ\text{C}$  for 30 min. After cooling, the sample was centrifuged at  $1200 \times g$  for 10 min, and the absorbance at 530 nm read on the Gilford spectrophotometer. The amount of TBA reactants was quantitated against a standard curve of malondialdehyde (1,1,3,3-tetramethoxypropane) ranging from 0.5 to 10  $\mu\text{M}$ .

Cyanmethemoglobin was measured in a 1.0-ml sample of culture medium containing KCN after lysing the red blood cells, as described in Fairbanks (28). The percentage of hemoglobin converted to cyanmethemoglobin was calculated using the formula:

$$[(\text{Control OD} - \text{Treated OD}) / (\text{Control OD})] \times 100$$

assuming no uncomplexed methemoglobin was present under the experimental conditions. This assumption appears valid because cyanmethemoglobin is readily formed (28,29).

Methemoglobin was determined on a 1.0-ml aliquot of the culture medium containing erythrocytes but no KCN by the clinical method described in Fairbanks (28). Methemoglobin was calculated as the percentage present in the sample compared with the total hemoglobin available for conversion to methemoglobin.

Thiocyanate was determined according to the method of Lundquist et al. [30] with modifications. Ion exchange resin, Lewatit MP 64 (Mobay Chemical Corp., Pittsburgh, PA), was washed as described except for omission of the drying step. The column method for separating thiocyanate was modified to a bulk extraction procedure. A 3-ml slurry of resin was put into a screw-cap test tube. Excess liquid was removed. The 0.10 N solution of NaOH, containing 0.10 ml of sample, was applied to the resin, and the tube was shaken for 10 min. Excess liquid was removed, and the resin was washed twice with 5.0 ml of water and three times with 5.0 ml of 0.10 N HCl, followed by three washes with 4.0-ml portions of 1.0 M NaClO<sub>4</sub> to collect the thiocyanate. The thiocyanate was quantitated by the modified Konig reaction as described (30).

Cyanide was determined as the difference in total Fe-reactive cyanide content in the Konig reaction and the component due to thiocyanate. In the experiment to determine unreacted cyanide in the reaction flasks, a 1.0-ml aliquot of the medium was placed in a centrifuge tube for immediate analysis and a second 1.0-ml aliquot was frozen for thiocyanate determination later, as described above. The tubes were centrifuged immediately in a clinical centrifuge to remove erythrocytes, and 0.10 ml was transferred into larger test tubes containing 4.9 ml of 0.10 N NaOH. The cyanide and thiocyanate were quantitated directly using the modified Konig reaction, as above, against a standard curve developed for KCN.

#### K<sup>14</sup>CN Experiments

Because hydrogen cyanide is gradually lost to the atmosphere in open flasks at 37°C (31), the tightness of the reaction flasks against such losses during incubations and the extent of partitioning of cyanide between aqueous and atmospheric phases were evaluated. In these experiments, K<sup>14</sup>CN (specific activity, ~ 1.90  $\mu$ Ci/ $\mu$ mole) was introduced by the standard procedure into the culture medium in separate flasks at 0.35, 1.1, and 5.0 mM (final concentrations). The radioactivity remaining in solution was determined as a function of the different steps in the experimental procedure and incubation time by scintillation counting. Approximately 5% of the radiolabel was lost from the solution after gassing, an additional 15% after the first 5 min of incubation, and no more than 5% thereafter over an additional 125 min of incubation, which included the brief opening of the gassing port for introduction of antidotes. These values were independent of the nominal dissolved concentration of KCN in the flasks. Nonradiolabelled KCN, therefore, was used in the reported studies, and the data are expressed as nominal [KCN] unless otherwise indicated. The actual dissolved cyanide concentration would be approximately 20% lower because of partitioning to the atmospheric phase; loss to the outside atmosphere is essentially negligible.

## RESULTS

### Cytotoxicity Experiments

The results of initial experiments of the effect of KCN concentration on steady-state ATP, urea synthesis, and intracellular LDH release from hepatocytes in monolayer cultures (18) were replicated and expanded for more precise quantitative values and to establish statistical significance for the changes induced. Figure 1 shows the mean values of steady-state ATP levels in hepatocytes for  $n = 4$  or 5 replicate experiments. KCN concentrations were varied from 0.10 to 2.5 mM for the incubation times previously used (10, 30, and 120 min) and for  $n = 3$  replicate experiments for earlier time points (2 and 5 min) to assess the rate of the observed changes more accurately. Only means are presented in the figure for ease of visualization. The complete data (means  $\pm$  SD) are appended as Table A-1.

As the cyanide concentration in the flask was increased, hepatocyte ATP levels decreased relative to control cell levels, as expected. The depression in ATP levels was statistically significant at  $\geq 0.25$  mM KCN, even at the earliest measurement (2 min). The change in ATP was maximal 10 min after addition of 0.10 and 0.25 mM KCN (70% and 47% of control levels, respectively), but at 1.0 mM KCN or higher, ATP levels continued to decrease appreciably over the next 110 min as the cells lost all capacity to resynthesize ATP. At 1.0 and 2.5 mM KCN and this incubation period, the ATP levels were less than 10% of control levels.

We interpret these results to indicate that cyanide ion binds initially to the cytochrome oxidase in mitochondria, inhibiting oxidative phosphorylation and resynthesis of ATP. At less than 1.0 mM cyanide, a new equilibrium level is reached, which is not "life-threatening" for cell survival; the cells can continue to function, though with some impairment (see discussion on urea synthesis below). At 1.0 mM or greater cyanide concentration, depression of mitochondrial ATP synthesis is too severe to permit maintenance of homeostasis. Other ATP pools in the cytoplasm deteriorate as the cells lose all capability to exclude the external medium and die.

Last year we reported that under the incubation conditions used in our experiments, ATP levels in control (untreated with KCN) hepatocytes decreased by over 20% in 2 hr (18). This observed change in control cell behavior was reproduced here (Figure 1). These changes are attributed in large part to the addition of the urea substrate, ornithine and  $\text{NH}_4\text{Cl}$  to the medium to permit accurate measurement of urea synthesis *in situ* (32, 33). When the urea substrates were omitted from the medium, the change in cellular ATP levels was  $< 15\%$  over a 2-hr incubation (data not shown).

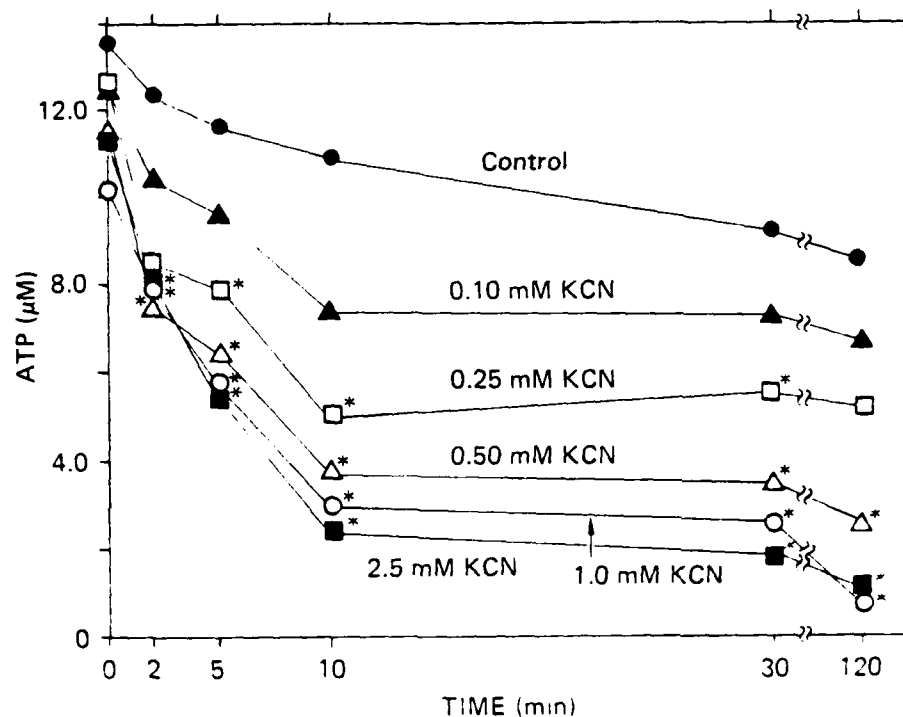


Figure 1

CONCENTRATION- AND TIME-DEPENDENT DEPRESSION OF HEPATOCYTE ATP LEVELS BY KCN. Rat hepatocytes were isolated by biopsy perfusion and cultured on collagen-coated plastic dishes ( $1.7 \times 10^6$  attached cells/dish) for 24 hr in 4.0 ml of Waymouth's 752/1 medium supplemented with 0.2% albumin and hormones [19]. To initiate the reaction, the culture medium was replaced by fresh medium containing the indicated concentration of cyanide and urea synthesis substrates, the dishes were transferred to air-tight reaction flasks, the atmosphere was replaced by gentle gassing through the ports with air:CO<sub>2</sub> (95:5), and the flasks were incubated at 37°C with gentle shaking. Separate flasks (in duplicate) were used for each sampling time: 0-, 2-, and 5-min data points are the means of three experiments; 10-, 30-, and 120-min data points are the means of five experiments, except at 2.5 mM KCN ( $n = 4$ ). Asterisk indicates significantly different from controls (no KCN) at  $p < 0.05$ .

Figure 2 shows the relationship of urea formation to KCN added to the flask. (Means and standard deviations for the data points in the figure are appended in Table A-2.) As KCN concentration was increased, urea formation was correspondingly decreased over time. The changes in urea content in the medium were not evident until sufficient production had occurred for detection ( $\geq 0.25$  mM KCN at 30 min and 0.10 mM KCN at 120 min). At the highest KCN concentrations (1.0 and 2.5 mM), there was no significant increase in urea in the medium between 10 and 120 min, indicating virtual shut-down of urea synthesis. The half maximal  $K_m$  of 1.4 mM for depression of urea accumulation was interpolated from a replot of the data used to construct Figure 2. This compares with an estimated  $K_m$  of 0.25 mM for ATP depression derived from plots of percent change in ATP at 10 min (Table A-1), when equilibrium appears to have been achieved, as a function of cyanide concentration.

In contrast to the early and sensitive responses of ATP and urea synthesis to cyanide, the data in Figure 3 show that LDH release from hepatocytes to the medium (evidence of complete loss of cell viability) is a late event. (Means and standard deviations for the data points in the figure are appended in Table A-3.) Thus, after 30 min statistically significant increases in LDH activity in the medium occurred at  $\geq 1.0$  mM KCN, but the magnitudes of the changes were small at all concentrations ( $< 10\%$  of the total cellular content). Appreciable LDH activity ( $> 10\%$  above background) was not seen in the medium at any KCN concentration until 120 min. The rate of appearance of LDH activity in the medium was appreciably greater in the second 120 min of incubation than in the first, analogous to what has been observed with other chemicals and indicating increased loss of cell viability (34,35). Because percent LDH release and percent cell viability assessed by other indicators (trypan blue exclusion and cell count) have been shown to correlate well for cyanide toxicity (23), it may be seen (Figure 3) that much higher cyanide concentrations (2.5 mM or above) and reaction times (120 to 240 min) are required for 30% or more of the cells to die than for an equivalent reduction in ATP levels or urea synthesis (Figures 1 and 2). The  $EC_{50}$ , slope of the dose-response curve, and in parentheses, the respective 95% confidence intervals, at 4 hr for LDH release were calculated from the data in Figure 3 to be 2.35 (7.59, 0.73) mM and 2.10 (6.53, 0.68), respectively.

We have also completed similar analyses of several other cytotoxicity parameters--lactate-to-pyruvate (L/P) ratio, intracellular reduced glutathione content, malondialdehyde formation [a measure of lipid peroxidation in the cells (36)], and release of AST and acid phosphatase enzymes [for monitoring mitochondrial and lysosomal membrane integrity, respectively (36)]. The results are presented in Table 1. Increases in L/P, which reflects the transition from aerobic to anaerobic pathways (37), were not detected in response to increasing KCN in the medium until 30 min of incubation, at which time the maximal increase compared with control cell flasks was two-fold. At 120 min, L/P in treated flasks was much more pronounced, peaking at 1.0 mM KCN and decreasing at higher concentrations (Table 1). This phenomenon was also observed in other studies and was ascribed to cessation of lactate and pyruvate synthesis in severely damaged hepatocytes (38,39).

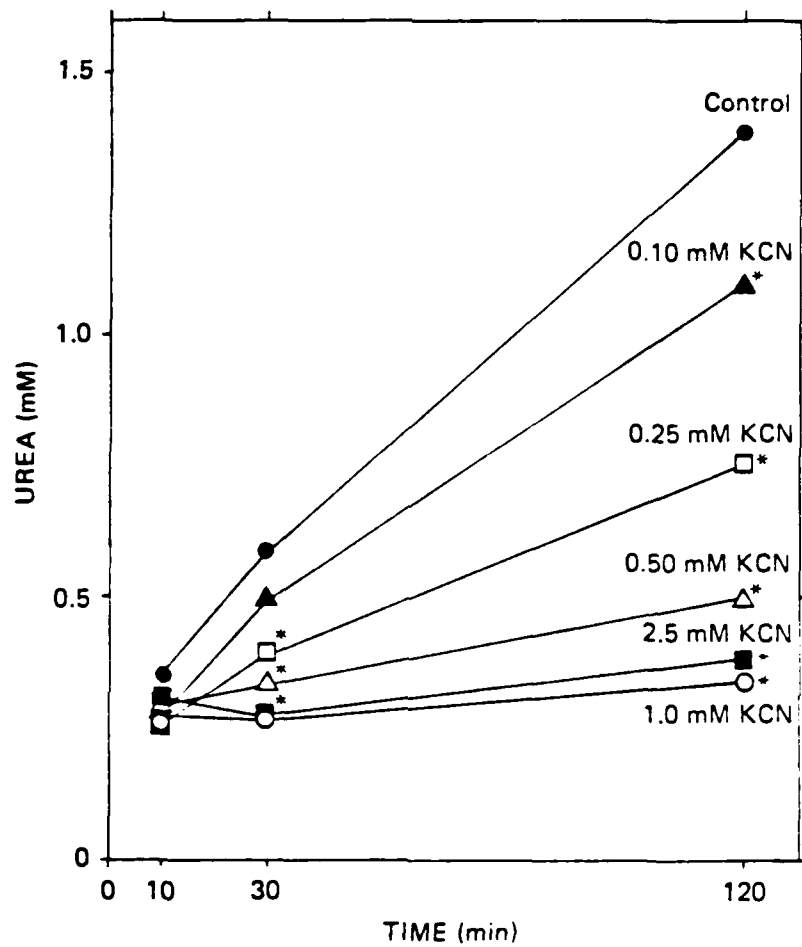


Figure 2

CONCENTRATION- AND TIME-DEPENDENT FORMATION OF UREA BY HEPATOCYTES. Experimental protocol is the same as described in the legend to Figure 1. Data points are the means for five experiments, except at 2.5 mM KCN ( $n = 4$ ). Asterisk indicates significantly different from controls (no KCN) at  $p < 0.05$ .

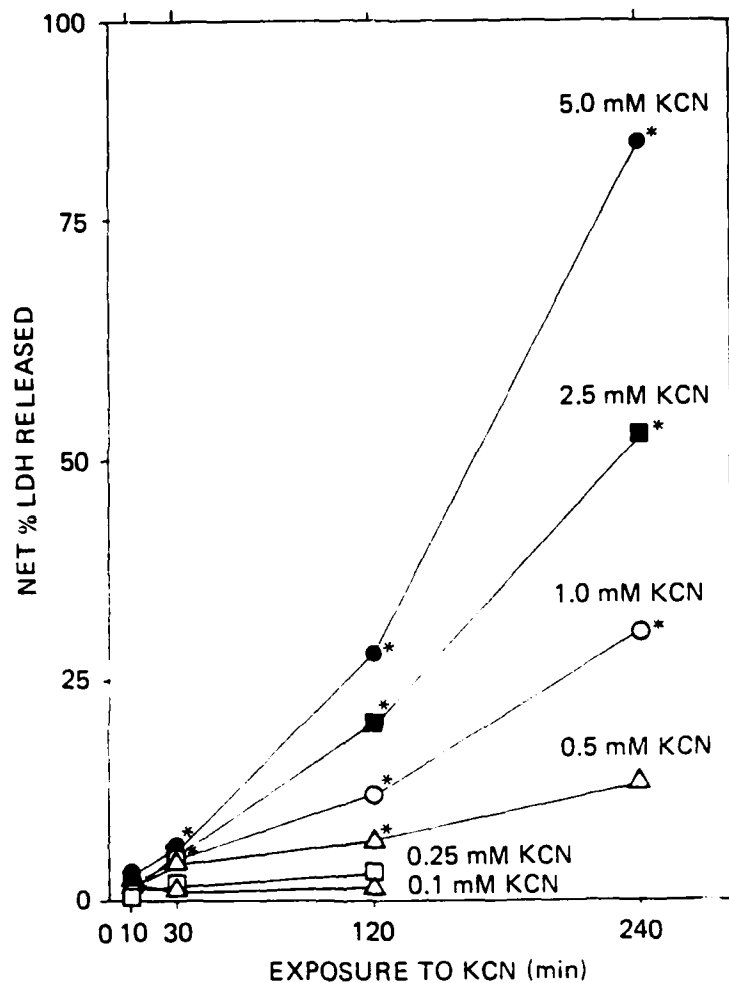


Figure 3

CONCENTRATION- AND TIME-DEPENDENT RELEASE OF LDH FROM HEPATOCYTES. Experimental protocol is the same as described in the legend to Figure 1. Data points are the means of four to eight experiments. Net % release is release due to KCN after subtracting out background control (no KCN) levels in medium. Mean total LDH available for release for the eight experiments reported was  $1510 \pm 184$  U/L. Asterisk indicates significantly different from controls (no KCN) at  $p < 0.05$ .



Table 1

FUNCTIONAL CAPABILITIES OF HEPATOCYTE CULTURES EXPOSED TO DIFFERENT KCN CONCENTRATIONS<sup>a</sup>

KCN (mM)	Incubation Time (min)	L/P Ratio <sup>b</sup>	GSH (mM) <sup>b</sup>	Enzyme Release <sup>c</sup>		
				AP (%)	LDH (%)	AST (%)
0.0	120	4.4 ± 0.35	1.9 ± 1.7 <sup>c</sup>	(8.5) <sup>d</sup>	(4.0)	(1.3)
0.5		21.0 ± 6.2	1.8 ± 1.9	0	3.3	1.2
1.0		29.4 ± 12.6	1.7 ± 1.7	0	1.6	0.4
2.5		20.8 ± 6.7	1.6 ± 1.4	0	4.0	2.6
5.0		12.7 ± 4.8	1.6 ± 1.4	9.3	13.1	1.9
0.0	240	--	0.8 (0.7, 0.9) <sup>c</sup>	(11.9)	(5.5)	(2.8)
0.5		--	1.6 ± 1.5	0	6.3	0.06
1.0		--	1.5 ± 1.1	23.1	41.3	4.2
2.5		--	2.7 ± 2.4	84.6	102.0	17.7
5.0		--	7.4 ± 10.5	113.5	104.0	26.6

<sup>a</sup> The experimental conditions and protocol are the same as those described in the legend to Figure 1, except for the omission of urea substrates.

<sup>b</sup> Means ± SD for at least  $n = 3$  experiments or mean with individual values in parentheses for  $n = 2$ .

<sup>c</sup> Values are net percent release calculated from the total in the medium divided by the total activity in the flask (cells + medium) after subtracting control cell release from each quantity × 100.

<sup>d</sup> Percent release to the medium from hepatocytes in control (no KCN added) flasks.

One parameter that continues to pose problems from a measurement standpoint is intracellular  $\text{Ca}^{2+}$  (18). Loss of  $\text{Ca}^{2+}$  storage capability in mitochondria and microsomes, which regulates cytosolic levels of the ion required for sustaining function, is postulated by several investigators to be a key factor leading to cell death (40-42). This postulate has been tested in a few cases with model cytotoxins by using uncouplers of oxidative phosphorylation as probes to quantify mitochondrial  $\text{Ca}^{2+}$ , the major storage site for this ion. Unfortunately, cyanide inhibits cytochrome oxidase and, in turn, the energy supplies for uncoupler action so that this approach cannot be used with this toxin. Development of another approach for measuring intracellular  $\text{Ca}^{2+}$  disposition is under study.

### Antidotes

We reported previously (18) that reversal of cellular ATP depression in hepatocytes was a practical means for evaluating antidote effectiveness and that classical antidotes such as sodium nitrite and sodium thiosulfate restored this indicator to control levels in the incubation system under appropriate experimental conditions. We have refined those conditions by developing an improved technique that allowed us to add higher levels of erythrocytes to the system (1.6 g Hb/dL). This permitted use of 1.0 mM KCN as the toxicant concentration, affording greater sensitivity in detecting and ranking antidote effectiveness with ATP. The antidotes were added to the reaction flasks 10 min after addition of cyanide, and recovery in ATP to control levels was assessed for up to 2 hr. The long incubation period was to ensure full recovery in the functional deficit (ATP depression) and to detect any possible delayed effects, which some antidotes are known to produce (4).

We repeated experiments on the reversal of cyanide-induced depression of ATP by sodium nitrite, sodium thiosulfate, and their combination, and evaluated 4-dimethylaminophenol (DMAP) and cobaltous chloride ( $\text{CoCl}_2$ ) for comparison. Sodium nitrite (5.0 mM), sodium thiosulfate (10 mM), or their combination substantially restored hepatocyte ATP levels within 60 min in the hepatocyte/erythrocyte system (Figure 4). [The means and standard deviations for ATP content and percent change relative to control (no KCN) flasks are appended in Tables A-4 and A-5.] The combination of sodium nitrite and sodium thiosulfate was the most effective of the three treatments. At 120 min, ATP levels in the experiments with sodium nitrite alone were lower than those in controls, possibly because of delayed hepatotoxicity associated with the high nitrite concentration. Neither the antidotes nor KCN (1.0 mM) affected erythrocyte ATP levels during the recovery period (data not shown).

Sodium nitrite was not effective in reversing ATP depression in hepatocytes in the absence of erythrocytes in the medium; hepatocyte ATP levels were 11% and 7% of those in control flasks (no KCN) at 120 min with and without 5.0 mM sodium nitrite present, respectively. Also, in the

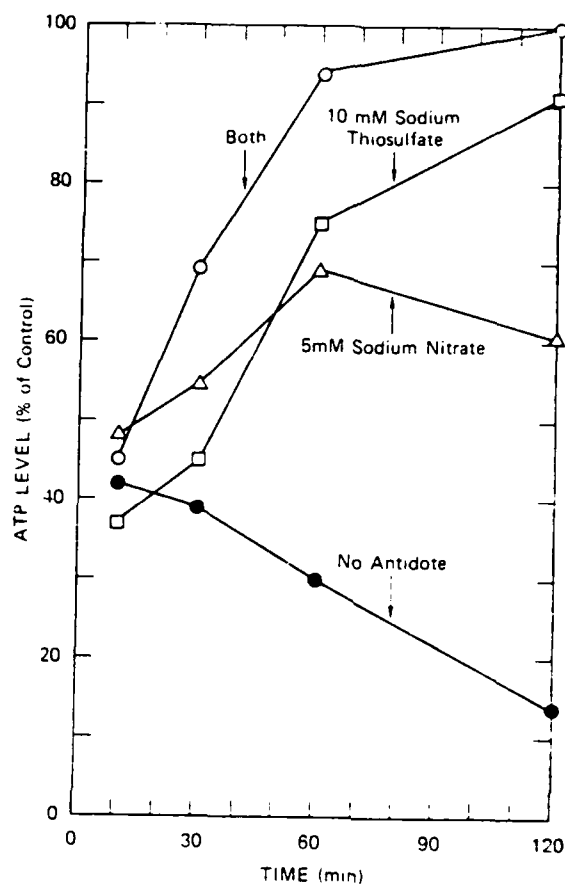


Figure 4

CAPABILITY OF SODIUM NITRITE, SODIUM THIOSULFATE, AND THEIR COMBINATION TO RESTORE ATP LEVELS IN THE COINCUBATION SYSTEM. Rat hepatocytes were isolated and cultured for 24 hr as described in Methods. To start the reaction, the culture medium was replaced by aspiration with fresh medium containing erythrocytes (1.6 g hemoglobin/dL) and KCN at the indicated concentration. The dishes were transferred to air-tight flasks and the atmosphere inside exchanged with air:CO<sub>2</sub> (95:5) for 1 min before stoppering the inlet port. After a 10-min incubation in a water bath at 37°C with gentle shaking, the antidote was added quickly through the inlet port and the reaction continued for up to 120 min. Separate flasks (in duplicate) were used for each sampling time. Data points are the means  $\pm$  SE of three experiments.

coincubation system in the absence of cyanide, 5.0 mM sodium nitrite produced a time-dependent increase in methemoglobin formation to 78% of total hemoglobin after 120 min (Figure 5). (The data used for Figure 5 are in appended Table A-6.) The agreement with published results is good (43,44). Essentially similar results had been obtained in an earlier experiment in which the erythrocyte content in the medium and KCN concentration were varied. Even though conversion to methemoglobin was 90% at one-fourth to one-half the erythrocyte content used here and 1.0 mM KCN, sodium nitrite did not reverse ATP depression (18), presumably because the binding capacity for cyanide in the flask was insufficient.

DMAP was also found to be effective in reversing cyanide-induced ATP depression in hepatocytes, but at less than one-tenth the concentration needed for sodium nitrite in similar experiments (Figure 6). (The data for the figure are appended in Tables A-7 and A-8.) By 10 min, ATP levels had recovered to more than 70% of their respective control (no KCN) levels at each DMAP concentration tested. At 0.50 mM DMAP, however, ATP levels after 120 min were noticeably lower than those at the other DMAP concentrations. In range-finding studies to determine appropriate test conditions for this antidote, 1.5 and 5.0 mM DMAP resulted in substantial (>75%) lowering of hepatocyte ATP levels and in LDH leakage by 30 min of incubation. These data indicate that DMAP is cytotoxic at 1.5 mM and above; the lower ATP levels noted at 0.50 mM may be a marginal cytotoxic response to DMAP evident at the longer (120 min) incubation time. These interpretations correspond well with other published data on the concentration range of DMAP producing cytotoxicity (45).

In these experiments, methemoglobin formation in the flasks containing no KCN was also quantitated. At 0.25 and 0.50 mM DMAP, the methemoglobin levels were 37 and 70% after 3 min and 62 and 98% after 10 min of incubation, respectively. Percentage methemoglobin remained essentially at these levels for an additional 110 min before termination of the experiment. The 0.50-mM DMAP concentration produced a more complete methemoglobinemia in a shorter period of time than did sodium nitrite at a ten-fold higher concentration (Figure 5). In contrast, ATP in the KCN-containing flasks did not recover fully to control levels at this DMAP concentration until 30 min. The observation that DMAP-induced methemoglobin formation is appreciably faster than ATP recovery (in contrast to sodium nitrite antidotal action) indicates that DMAP is optimally effective and that other factors--dissociation of the cytochrome oxidase-cyanide complex, migration from the inhibitory site, and/or complexation with methemoglobin--are rate-limiting. Additional experiments are required to substantiate this interpretation.

Based on these experiments, the following protocol was adopted for initial screening of the antidotes for therapeutic potential. Hepatocytes were incubated for 10 min with 1.0 mM KCN (to maximize sensitivity and facilitate handling of up to 56 flasks in timed-sequence experiments), at which time the antidotes were added directly to the flasks and the incubations continued for an additional 60 min. Sodium nitrite, sodium thiosulfate, a combination of these antidotes,  $\text{CoCl}_2$ , and DMAP were

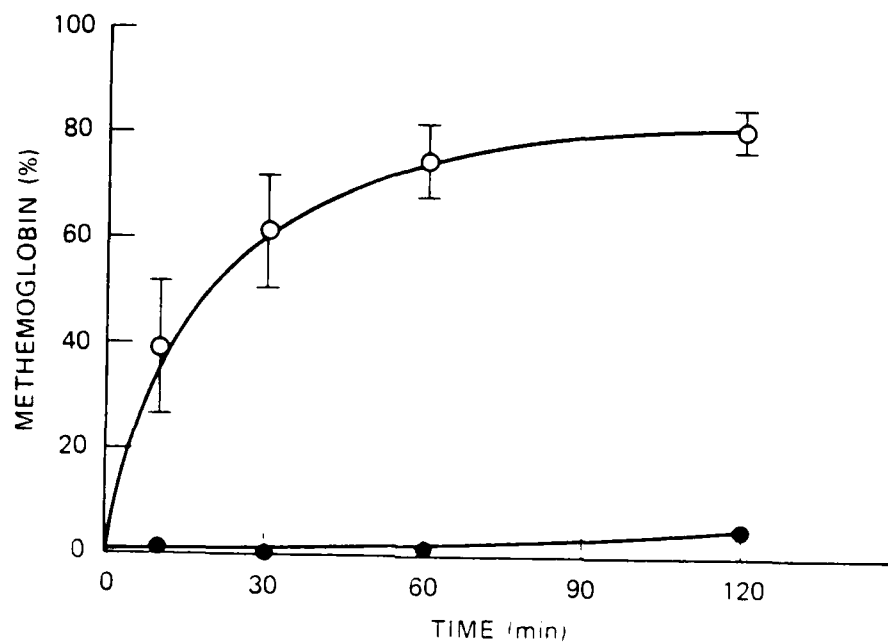


Figure 5

METHEMOGLOBIN FORMATION INDUCED BY SODIUM NITRITE IN THE COINCUBATION SYSTEM. Rat hepatocytes were isolated by biopsy perfusion and cultured on collagen-coated plastic dishes for 24 hr in 4.0 ml of Waymouth's 752/1 medium supplemented with 0.2% albumin and hormones ( $1.6 \times 10^6$  attached cells/dish) [19]. To start the reaction, the medium was replaced by aspiration with fresh medium containing erythrocytes (1.6 g hemoglobin/dL) and the dishes transferred to air-tight flasks. No KCN was added. After a 10-min incubation in a water bath at 37°C with gentle shaking, the antidote was added quickly through the inlet port and the reaction continued for up to 120 min. Separate flasks (in duplicate) were used for each sampling time. Data are the means  $\pm$  SE of three experiments, except at 60 min  $n = 5$ , for flasks containing 5.0 mM NaNO<sub>2</sub> (○) and no NaNO<sub>2</sub> (●).

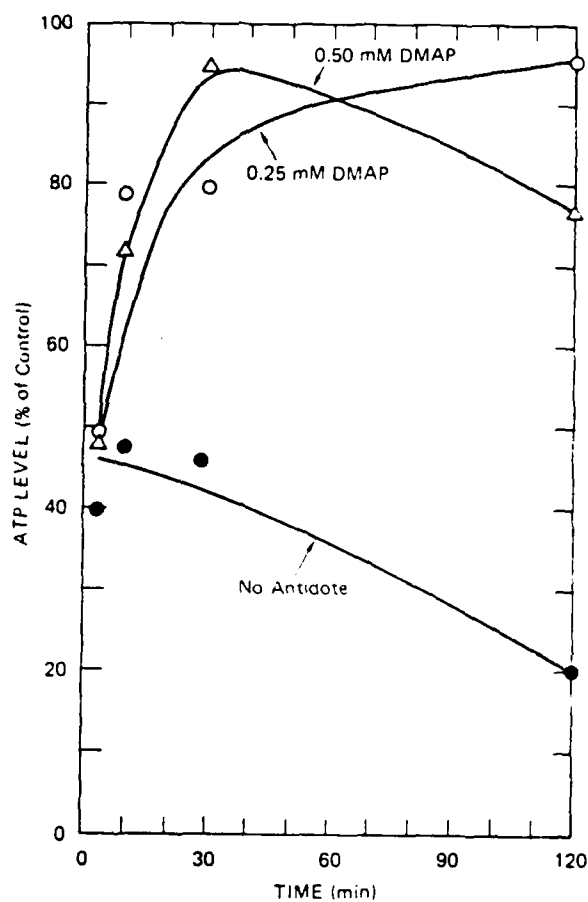


Figure 6

CAPABILITY OF DMAP TO RESTORE ATP LEVELS IN THE COINCUBATION SYSTEM. Experimental protocols and sampling times are as described in the legend to Figure 4. Erythrocyte level was equivalent to 1.6 g hemoglobin/dL. ATP was quantitated in hepatocytes after removal of erythrocytes from reaction flasks by aspiration. ATP levels in flasks containing 0 and 1.0 mM KCN were 5.6 and 2.9  $\mu$ M at 30 min and 5.4 and 1.1  $\mu$ M at 120 min, respectively.

Table 2

## REVERSAL OF ATP SUPPRESSION IN HEPATOCYTES BY CYANIDE ANTIDOTES

Antidote <sup>a</sup> (mM)	ATP Level (10 <sup>-5</sup> M)		ATP % of Control <sup>b</sup>	% Methemoglobin <sup>c</sup>	% Cyan- methemoglobin <sup>d</sup>
	No KCN	1 mM KCN			
None	1.19 ± 0.25 <sup>d</sup>	0.24 ± 0.11	19.6 ± 6.2	2.3 ± 2.0	
DMAP 0.05	1.26 ± 0.24	0.42 ± 0.08	33.9 ± 4.6	27.0 ± 6.2	64.3 ± 5.8
0.10	1.20 ± 0.29	0.71 ± 0.24	58.9 ± 10.2	28.9 ± 4.2	85.3 (75.1, 95.5)
0.25	1.25 ± 0.24	0.81 ± 0.20	64.5 ± 5.7	48.9 ± 13.4	83.3 ± 10.8
CoCl <sub>2</sub> 0.10	1.22 ± 0.29	0.42 ± 0.31	32.3 ± 18.4	0.4 ± 0.7	9.1 (0.0, 18.2)
0.25	1.16 ± 0.29	0.98 ± 0.23	85.3 ± 10.0	0.0 ± 0.0	3.8 ± 6.6
NaNO <sub>2</sub> 2.0	1.16 ± 0.17	0.36 ± 0.12	30.7 ± 6.2	27.6 ± 16.7	38.5 (38.4, 38.6)
5.0	1.16 ± 0.23	0.52 ± 0.19	43.9 ± 8.9	56.7 ± 24.8	84.6 ± 7.4
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 4.0	1.16 ± 0.24	0.38 ± 0.04	33.5 ± 8.1	0.3 ± 0.5	19.3 (0.0, 38.6 <sup>e</sup> )
10.0	1.12 ± 0.30	0.66 ± 0.23	62.3 ± 26.5	0.2 ± 0.3	3.0 ± 5.2
NaNO <sub>2</sub> + Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 2.0	1.19 ± 0.20	0.67 ± 0.04	57.5 ± 9.5	28.3 ± 14.0	41.6 (83.1, 0.0)
4.0					
NaNO <sub>2</sub> + Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 5.0	1.14 ± 0.22	0.79 ± 0.12	70.4 ± 8.9	55.7 ± 20.4	60.6 (86.5, 34.1)
4.0					
NaNO <sub>2</sub> + Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 2.0	1.06 ± 0.29	0.78 ± 0.15	77.1 ± 20.0	30.8 ± 19.8	56.9 (81.9, 31.8)
10.0					
NaNO <sub>2</sub> + Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 5.0	1.14 ± 0.15	0.84 ± 0.09	73.8 ± 2.7	61.9 ± 32.5	84.1 ± 27.5
10.0					

<sup>a</sup> Antidote added 10 min after KCN. KCN not removed. Experimental protocols are described in Methods.

<sup>b</sup> No KCN added. Measured 60 min after addition of antidote.

<sup>c</sup> Approximately 1.6 g hemoglobin/dL per sample. Methemoglobin measured in no-KCN treated flasks.

<sup>d</sup> Data are means ± SD of n = 3 experiments, or, where individual values are given in parentheses, n = 2.

<sup>e</sup> Spurious; due to experimental error or variability.

evaluated. The results with the five antidotal treatments are summarized in Table 2. DMAP, cobalt(II) chloride, sodium nitrite, sodium thiosulfate, and combinations of the last two were all effective in an apparently dose-related manner in reversing ATP suppression. The antidote concentrations for ATP recovery to 50% of the control (no KCN) level, which can be estimated from the data, indicates a relative effectiveness ranking of  $\text{DMAP} > \text{CoCl}_2 \gg \text{Na}_2\text{S}_2\text{O}_3 \approx \text{NaNO}_2$ . In general, the combination of sodium thiosulfate and sodium nitrite appears to be more effective than the separate antidotes in restoring ATP levels to near control flask values.

The mechanism of action of some of the antidotes was inferred from the data in the column on percent methemoglobin formation (Table 2). These measurements were made on erythrocytes in the combined cell system in the absence of KCN in the medium. DMAP and sodium nitrite (alone or in combination with thiosulfate) produced substantial methemoglobin in the combined cell system, whereas cobalt(II) chloride and sodium thiosulfate did not. In addition, the presence of sodium thiosulfate had no appreciable effect on the percentage of methemoglobin formed by sodium nitrite in this system, consistent with the basically different mechanisms thought to apply for these two antidotes.

When KCN (1.0 mM) was present in the medium, cyanmethemoglobin in the erythrocytes could be quantitated spectrophotometrically after the additional 60-min incubation with antidotes. The results are shown in the far right-hand column in Table 2. As expected from the results with no KCN present, flasks containing antidotes that produce methemoglobin (DMAP and sodium nitrite) had high levels of cyanmethemoglobin in the presence of KCN. The levels of cyanmethemoglobin quantitated for these antidotes were higher than those for methemoglobin in the absence of KCN, which may reflect a more stable complex with KCN or may result from analytical differences. Antidotes [sodium thiosulfate and cobalt(II) chloride] that did not produce methemoglobin in the "no-KCN" flasks produced little cyanmethemoglobin with KCN present.

We discussed earlier that sodium nitrite required the presence of erythrocytes in the test system for effective reversal of cyanide-induced ATP suppression, as expected if methemoglobin formation is involved in the mechanism of sodium nitrite action. We conducted similar experiments with sodium thiosulfate (i.e., plus or minus erythrocytes in the system) and varied albumin content in the medium to gain some insight into how sodium thiosulfate reverses cyanide toxicity (Figure 4; Table 2). Sodium thiosulfate is thought to convert cyanide *in vivo* to sodium thiocyanate, a much less toxic ion, by means of rhodanese in liver cells and/or albumin in serum (46).

Table 3 presents the ATP results and Table 4 expresses those data as the percentage of ATP obtained at the same sampling times. As shown in Table 4, in the absence of erythrocytes, sodium thiosulfate produced a substantial restoration in hepatocyte ATP toward control levels in the KCN-containing flasks, but the effect was delayed until 60 min.



Table 3

INFLUENCE OF ALBUMIN AND ERYTHROCYTES  
ON THIOSULFATE-MEDIATED REVERSAL OF KCN TOXICITY

KCN (mM)	Albumin (%)	RBC (g hemo- globin/dL)	Thiosulfate <sup>a</sup> (mM)	ATP Level ( $10^{-5}$ M)		
				30 Min <sup>b</sup>	60 Min <sup>b</sup>	120 Min <sup>b</sup>
0	0.2	0	0	0.84 $\pm$ 0.24 <sup>c</sup>	0.75 $\pm$ 0.18 <sup>d</sup>	0.73 $\pm$ 0.21 <sup>d</sup>
0	0.2	0	10	0.71 $\pm$ 0.28	0.67 $\pm$ 0.15	0.60 $\pm$ 0.10
0	0.2	1.6	0	0.92 $\pm$ 0.28	0.82 $\pm$ 0.24	0.69 $\pm$ 0.08
0	0.2	1.6	10	0.83 $\pm$ 0.33	0.72 $\pm$ 0.10	0.62 $\pm$ 0.09
1	0.2	0	0	0.17 $\pm$ 0.17	0.05 $\pm$ 0.02	0.10 $\pm$ 0.08
1	0.2	0	10	0.19 $\pm$ 0.06	0.23 $\pm$ 0.14	0.52 $\pm$ 0.09
1	0.2	1.6	0	0.18 $\pm$ 0.08	0.13 $\pm$ 0.04	0.16 $\pm$ 0.06
1	0.2	1.6	10	0.39 $\pm$ 0.13	0.44 $\pm$ 0.03	0.58 $\pm$ 0.12
0	5.0	0	0	0.67 $\pm$ 0.29	0.64 $\pm$ 0.14	0.57 $\pm$ 0.13
0	5.0	0	10	0.59 $\pm$ 0.17	0.50 $\pm$ 0.07	0.46 $\pm$ 0.10
0	5.0	1.6	0	0.74 $\pm$ 0.32	0.60 $\pm$ 0.10	0.59 $\pm$ 0.15
0	5.0	1.6	0	0.69 $\pm$ 0.32	0.56 $\pm$ 0.08	0.55 $\pm$ 0.10
1	5.0	0	0	0.16 $\pm$ 0.04	0.13 $\pm$ 0.07	0.17 $\pm$ 0.06
1	5.0	0	10	0.20 $\pm$ 0.04	0.30 $\pm$ 0.09	0.42 $\pm$ 0.10
1	5.0	1.6	0	0.26 $\pm$ 0.06	0.16 $\pm$ 0.02	0.23 $\pm$ 0.08
1	5.0	1.6	10	0.39 $\pm$ 0.15	0.48 $\pm$ 0.11	0.54 $\pm$ 0.10

<sup>a</sup> Thiosulfate added 10 min after KCN. KCN not removed. Experimental protocols are described in Methods.

<sup>b</sup> After thiosulfate addition.

<sup>c</sup> Mean  $\pm$  SD of three experiments for values in this column.

<sup>d</sup> Mean  $\pm$  SD of four experiments for values in this column.

Table 4

PERCENTAGE OF ATP RECOVERED AFTER  
THIOSULFATE-MEDIATED REVERSAL OF KCN TOXICITY

Albumin (%)	RBC <sup>a</sup>	Thiosulfate (10 mM)	ATP Level (% of Corresponding "No KCN" Control) <sup>b</sup>		
			30 min <sup>c</sup>	60 Min <sup>c</sup>	120 Min <sup>c</sup>
0.2	No	No	20.3 ± 7.7 <sup>d</sup>	12.3 ± 10.4 <sup>e</sup>	14.5 ± 12.0 <sup>e</sup>
	No	Yes	27.4 ± 9.2	38.5 ± 25.8	86.0 ± 3.7
	Yes	No	20.4 ± 1.6	17.5 ± 9.5	22.5 ± 10.4
	Yes	Yes	47.6 ± 3.9	62.3 ± 6.2	92.3 ± 7.4
5.0	No	No	24.4 ± 5.2	21.5 ± 13.7	30.8 ± 10.4
	No	Yes	33.7 ± 4.0	62.0 ± 21.8	91.0 ± 8.5
	Yes	No	37.3 ± 9.8	26.8 ± 7.0	43.5 ± 14.1
	Yes	Yes	57.9 ± 8.7	84.3 ± 12.8	99.3 ± 11.5

<sup>a</sup> Approximately  $3 \times 10^3$  RBCs, or 1.6 g hemoglobin equivalents/dL, when present, in culture dishes containing  $\sim 1.5 \times 10^6$  hepatocytes.

<sup>b</sup> Calculated from data in Table 3.

<sup>c</sup> After thiosulfate addition.

<sup>d</sup> Mean ± SD of three experiments for values in this column.

<sup>e</sup> Mean ± SD of four experiments for values in this column.

Increasing the albumin content from 0.2% to 5.0% in the medium led to a slight further enhancement in ATP recovery. Possible explanations for these observations are: (1) rhodanese activity in the hepatocytes becomes prominent as sodium thiosulfate uptake by the cells, and particularly mitochondria, reaches steady-state levels; (2) sulfane sulfur is released from thiosulfate at the membrane surface or in the cytoplasm and is converted by hepatic enzymes to elemental sulfur for uptake by albumin in the medium, followed by catalytic transfer to cyanide to form thiocyanate, as discussed by Westley et al. (46); or (3) both.

Surprisingly, we also found (Table 4) that the presence of erythrocytes in the medium accelerated the recovery in ATP levels. After 30 min of incubation with 10 mM sodium thiosulfate in the combined system, ATP levels had returned to approximately 50% of control (no KCN) levels, an appreciably higher level of recovery than when erythrocytes were absent.

Because sodium thiosulfate is thought to be a substrate for enzymes that convert  $CN^-$  to  $CNS^-$ , the latter ion was quantitated in this experimental system. The results are summarized in Table 5. They show that the addition of sodium thiosulfate to cyanide-containing flasks with hepatocytes only increased the amounts of  $CNS^-$  10- to 30-fold or more over those in control flasks containing no sodium thiosulfate. The addition of erythrocytes to the flasks resulted in a further 60% to 70% increase in  $CNS^-$  after 60 min of incubation. Increasing the albumin content in the medium from 0.2% to 5.0% increased the percentage of conversion of  $CN^-$  to  $CNS^-$ , though not as much. At 120 min, there was less  $CNS^-$  in most flasks than at 60 min, a possible result of further reactions of this ion with components in the cells or in the medium. Overall, however, in short-term incubation (1) the conversion of  $CN^-$  to  $CNS^-$  in sodium thiosulfate-containing flasks appears to be the mechanism by which this antidote reverses cyanide-induced ATP suppression, and (2) erythrocytes as well as hepatocytes and/or albumin can participate in this process. Toida (47) has reported recently that rat erythrocytes can catalyze the conversion of  $CN^-$  to  $CNS^-$  with sodium thiosulfate.

In addition to these experiments on the mechanism of action of sodium nitrite and sodium thiosulfate, we tested the hypothesis that methylene blue might interfere with the former's effectiveness by preventing methemoglobin formation in the combined cell system we use (16). Specifically, we conducted two experiments to evaluate the effectiveness of methylene blue on sodium nitrite-induced reversal of cyanide toxicity to rat hepatocytes in the presence and absence of sodium thiosulfate. Methylene blue at either 0.50 or 1.0 mM in the medium had no effect on the percentage of methemoglobin produced by sodium nitrite after 60 min or on the percentage of cyanmethemoglobin formed when cyanide was also present in the medium. Methylene blue at these concentrations also produced modest levels of methemoglobin (58%) and considerable cyanmethemoglobin (79%) in the absence of sodium nitrite, resulting in a slight improvement in hepatocyte ATP levels. Thus, we were not able to demonstrate that methylene blue could antagonize methemoglobin formation by sodium nitrite

Table 5

THIOSULFATE-MEDIATED CONVERSION OF CYANIDE  
TO THIOCYANATE IN THE HEPATOCYTE/ERYTHROCYTE SYSTEM

Albumin (%) in Medium)	RBC <sup>a</sup>	Sodium Thiosulfate <sup>b</sup> (10 mM)	CNS <sup>-</sup> Level <sup>c</sup> (mM) After:		
			30 Min <sup>d</sup>	60 Min <sup>d</sup>	120 Min <sup>d</sup>
0.2	No	No	0.028 ± 0.027(4) <sup>e</sup>	0.020 ± 0.020(4)	0.037 ± 0.056(4)
	No	Yes	0.311 ± 0.038(3)	0.491 ± 0.062(4)	0.649 ± 0.077(4)
	Yes	No	0.019 ± 0.014(3)	0.023 ± 0.010(3)	0.050 ± 0.041(3)
	Yes	Yes	0.523 ± 0.319(4)	0.773 ± 0.062(4)	0.580 ± 0.136(3)
5.0	No	No	0.015 ± 0.009(4)	0.020 ± 0.009(4)	0.023 ± 0.007(4)
	No	Yes	0.393 ± 0.096(3)	0.600 ± 0.063(4)	0.502 ± 0.090(4)
	Yes	No	0.023 ± 0.015(3)	0.034 ± 0.006(2)	0.034 ± 0.022(3)
	Yes	Yes	0.471 ± 0.249(4)	0.648 ± 0.074(3)	0.483 ± 0.116(3)

<sup>a</sup> ~ 3 × 10<sup>5</sup> RBCs, or 1.6 g hemoglobin equivalents/dL, when present, in culture dishes containing ~ 1.5 × 10<sup>6</sup> hepatocytes.

<sup>b</sup> Thiosulfate added 10 min after KCN.

<sup>c</sup> Data are from same experiments described in footnote to Table 3.

<sup>d</sup> After thiosulfate addition.

<sup>e</sup> Mean ± SD, with number of experiments in parentheses.

or interfere with sodium nitrite-induced recovery of ATP levels in hepatocytes as a target cell for cyanide toxicity by this mechanism. However, methylene blue under these conditions did lower hepatocyte ATP to ~ 40% of control levels, probably because of dissipation of cellular energy through redox cycling. Therefore, methylene blue needs to be examined at lower concentrations for a more definitive conclusion.

In addition to these experiments, we evaluated cobalt(II) bis-bipyridyl, cobalt(II) chloride, and hydroxycobalamin in a prophylactic protocol in which these agents were added to the combined hepatocyte/erythrocyte system 10 min before the addition of cyanide. At concentrations of 0.5 and 1.0 mM, each was protective against cyanide-induced hepatotoxicity for a subsequent 120 min (Table 6). No substantial methemoglobin formation occurred in erythrocytes exposed to the cobalt compounds in control flasks during the incubation, and no clear signs of toxicity were apparent in either hepatocytes or erythrocytes in the absence of cyanide (Table A-9; ATP levels were also unchanged, data not shown).

#### Hepatocytes from Other Species

Hepatocytes isolated from a beagle, and from two human liver specimens were also used to evaluate cyanide action before conducting antidotal studies with hepatocytes from these species. In hepatocytes from the beagle, ATP levels were depressed and LDH release was increased as a function of increasing KCN concentration in the medium (Tables 7 and 8). The depression of ATP was greater than we have experienced with rat hepatocytes at the same KCN concentration, but LDH release as a function of time was generally less. Replicate experiments are needed before any definitive conclusions on susceptibility relative to other species can be drawn.

In addition to the above experiment with beagle hepatocytes, two different human liver specimens became available from heart-lung transplants performed at Stanford University Hospital. The first of these was used to demonstrate that hepatocytes could be isolated by our procedures and kept viable in culture for 24 hr or longer. The second specimen was used to test KCN for cytotoxicity. The effects of KCN on hepatocyte ATP levels and LDH release in this experiment are given in Tables 9 and 10. Compared with the data in Tables 7 and 8, the results generally suggest that human hepatocytes may be slightly less susceptible to KCN toxicity than dog hepatocytes. Opportunities to repeat these experiments and evaluate antidotes with additional biopsy specimens are being sought.

Table 6

ANTIDOTAL EFFECTIVENESS OF COBALT COMPOUNDS FOR PREVENTING CYANIDE-INDUCED ATP DEPRESSION IN THE COMBINED HEPATOCYTE/ERYTHROCYTE SYSTEM<sup>a</sup>

Antidote (mM)		ATP Levels <sup>b</sup> ( $10^{-5}$ M) at:					
		10 Min		30 Min		120 Min	
		0 KCN	1 mM KCN	0 KCN	1 mM KCN	0 KCN	1 mM KCN
None		0.73	0.41	0.63	0.31	0.62	0.22
Cobalt(II)	0.5	0.70	0.72	0.61	0.61	0.53	0.45
bis-bipyridyl	1.0	0.65	0.76	0.70	0.61	0.48	0.45
Cobalt(II)	0.5	0.74	0.70	0.76	0.61	0.52	0.53
chloride	1.0	0.64	0.67	0.63	0.62	0.53	0.48
Hydroxy-	0.5	0.56	0.68	0.67	0.55	0.56	0.44
cobalamin	1.0	0.71	0.69	0.68	0.63	0.55	0.51

<sup>a</sup> Cobalt compounds at the concentrations indicated above were added to hepatocytes and erythrocytes in the test system described in Methods; KCN was added 10 min later. Samples were assayed for hepatocyte ATP levels at the times shown after KCN addition.

<sup>b</sup> Each data point is mean of two replicates.

Table 7

ATP LEVELS IN DOG HEPATOCYTES AS A FUNCTION  
OF TIME AT DIFFERENT KCN CONCENTRATIONS

KCN (mM)	ATP Levels <sup>a</sup> ( $10^{-5}$ M) at:			
	10 Min	30 Min	120 Min	240 Min
0	0.97	0.88	0.60	0.92
0.5	0.30	0.12	0.16	0.11
1.0	0.13	0.052	0.037	0.0033
2.5	0.17	0.022	0.0046	0.0011
5.0	0.070	0.023	0.0029	0.0015
10.0	0.060	0.015	0.0024	0.0016

<sup>a</sup> Each data point is the mean of three replicates. Experimental protocols with dog hepatocytes were the same as described in Figure 1 with rat hepatocytes except that ornithine and  $\text{NH}_4\text{Cl}$  were not added to the medium.

Table 3

LDH RELEASE IN DOG HEPATOCYTES AS A FUNCTION  
OF TIME AT DIFFERENT KCN CONCENTRATIONS

KCN (mM)	LDH Release <sup>a</sup> (%) at:		
	30 Min	120 Min	240 Min
0.5	0.17	0	0.84
1.0	0	0.33	9.06
2.5	0	2.01	71.48
5.0	0.50	7.69	41.44
10.0	0.33	9.53	53.19

<sup>a</sup> Above background LDH release in control cells (no KCN added). Each data point is the mean of three replicates. Same experiment as described in Table 7.

Table 9

ATP LEVELS IN HUMAN HEPATOCYTES AS A FUNCTION  
OF TIME AT DIFFERENT KCN CONCENTRATIONS

KCN (mM)	ATP Levels <sup>a</sup> ( $10^{-5}$ M) at:		
	10 Min	120 Min	240 Min
0	0.62	0.69	0.64
0.5	0.27	0.048	0.034
1.0	0.25	0.0086	0.0057
2.5	0.20	0.0024	0.00093
5.0	0.21	0.0019	0.00047
10.0	0.13	0.0015	0.00032

<sup>a</sup> Each data point is the mean of two replicates. Experimental protocols with human hepatocytes were the same as described in Figure 1 with rat hepatocytes except that ornithine and  $\text{NH}_4\text{Cl}$  were not added to the medium.

Table 10

LDH RELEASE IN HUMAN HEPATOCYTES AS A FUNCTION  
OF TIME AT DIFFERENT KCN CONCENTRATIONS

KCN (mM)	LDH Released <sup>a</sup> (%) at:		
	10 Min	120 Min	240 Min
0.50	0	0.0	15.0
1.0	0	3.5	9.0
2.5	0	5.4	28.0
5.0	0	5.4	31.1
10.0	0	6.7	51.4

<sup>a</sup> Above background LDH release in control cells (no KCN). Each data point is the mean of two replicates. Same experiment as described in Table 9.



## CONCLUSIONS

The coincubation approach appears to be a particularly promising one for the study of cyanide and antidotal action at the cellular level and for the screening of newer antidotes for evidence of potential efficacy. The action of cyanide on hepatocytes as the target cell in the system we are currently using is consistent with what is expected if inhibition of cytochrome oxidase is the initiating event. ATP is depressed as would be expected from the inability of mitochondria to resynthesize the nucleotide. Urea synthesis, which depends on the energy state of the cell, is inhibited, and L/P rises during the transition from aerobic to anaerobic processes as the cells synthesize more ATP. When ATP depression is severe (at  $\geq 0.50$  mM cyanide in our experiments) and continues for a long time (1 hr or longer), the cells lose their capability for maintaining homeostasis (48) and intracellular enzymes are lost to the external medium. Erythrocytes remain intact during this period; no free hemoglobin appears in the medium.

Although these observations do not exclude other cytotoxicity mechanisms (49), two alternatives were evaluated and dismissed because of a lack of supporting data. Thus, GSH levels were not depressed and lipid peroxidation was not detected in hepatocytes treated with cyanide up to the point of cell death. The possibility that cyanide might form cyanohydrins with Schiff bases, disrupting intermediary metabolism (6), was considered. If this were true, one would expect a depression in total AST, which enzyme requires pyridoxal-5'-phosphate as a cofactor at cytotoxic concentrations of cyanide. In an earlier study with rat hepatocyte suspensions (23), we acquired data addressing this point. Thus, total AST (medium + cells) was depressed 20 and 30% after 2- and 5-hr incubations of the hepatocytes with 1.0 mM KCN and only 5% at most with 0.10 mM KCN in the medium; total LDH was not appreciably changed. This apparently selective action on AST is tentatively attributed to cyanohydrin formation with the Schiff base cofactor at the active site of the enzyme (50). However, because the change in total AST was far less than LDH release (81%) at 5 hr in those experiments, the change appears to be relatively minor and unrelated to the mechanism of cell death. It must be cautioned that this interpretation is tentative, because of dilution of the aliquot and addition of cofactor itself in performing the assay (51), which may result in some reversion of the cyanide-induced inhibition.

The coincubation system also appears to be potentially useful for preliminary screening of antidotes against cyanide action and for mechanistic studies. A requirement for screening is that the system should predict relative effectiveness with reasonable confidence.  $\text{NaNO}_2$ , DMAP,  $\text{CoCl}_2$ , and  $\text{Na}_2\text{S}_2\text{O}_3$ , known antagonists of cyanide toxicity in animal studies, reversed cyanide-induced ATP depression in the hepatocytes in a

concentration-dependent manner. The relative potency of these antidotes in the system is inferred from the data in Table 2 to be, on a millimolar basis,  $\text{DMAP} > \text{CoCl}_2 \gg \text{NaNO}_2 \approx \text{Na}_2\text{S}_2\text{O}_3$ . The ranking is qualitatively similar to the relative potency of the antidotes in vivo for preventing lethality in mice, the only species for which published data on these four antidotes were available for comparison (15, 52, 53). These data indicate that the following ip doses (in mmol/kg) prevent lethality:  $\text{CoCl}_2$ , 0.08-0.25; DMAP, 0.29;  $\text{NaNO}_2$ , 1.0-1.4; and  $\text{Na}_2\text{S}_2\text{O}_3$ , 6.3-8.0. This compares favorably with the in vitro potency ranking (Table 11). The combination of  $\text{Na}_2\text{S}_2\text{O}_3$  and  $\text{NaNO}_2$  was more effective than either alone, as has been demonstrated in vivo (54).

Table 11

COMPARISON OF ANTIDOTE EFFECTIVENESS  
IN VITRO AND IN VIVO

<u>Antidote</u>	<u>In Vitro<sup>a</sup></u> <u>(mM)</u>	<u>In Vivo<sup>b</sup></u> <u>(mmol/kg)</u>
$\text{CoCl}_2$	0.10-0.25	0.08-0.25
DMAP	0.10	0.29
$\text{NaNO}_2$	2.0-5.0	1.0-1.4
$\text{Na}_2\text{S}_2\text{O}_3$	4.0-10.0	6.3-8.0

<sup>a</sup> Coincubation system using rat hepatocytes and erythrocytes. Concentration or range at which a statistically significant recovery in ATP levels occurred.

<sup>b</sup> Doses given ip to mice (15,52,53).

Mechanistic studies on these antidotes have led to the following conclusions.

The results with  $\text{NaNO}_2$  and DMAP indicate quite conclusively that the reversal of ATP depression by cyanide is dependent on the capability of the antidotes to form methemoglobin, based on the following: (1) The antidotes produced substantial methemoglobin in the system with KCN absent, (2) erythrocytes were required in the system for reversal of

cyanide-induced ATP depression to occur, (3) cyanmethemoglobin was produced at approximately stoichiometric levels with the quantity of KCN originally added, and (4) in the absence of  $\text{Na}_2\text{S}_2\text{O}_3$ , thiocyanate formation was insignificant ( $< 1.0\%$ ). These results parallel those of earlier investigators showing that these antidotes also reverse cytochrome oxidase inhibition by cyanide in vitro and in vivo (55-58). The results here do not exclude the possibility that other mechanisms may also be involved in vivo, as has been proposed for  $\text{NaNO}_2$  (6). However, the ability to correlate methemoglobin levels with protection against lethality, in this case in mice and dogs, argues against this point of view and in support of the methemoglobinemia hypothesis (58,59).

In the case of  $\text{CoCl}_2$ , omission of erythrocytes from the medium did not prevent antagonism by  $\text{CoCl}_2$ , and the antidote produced no cyanmethemoglobin in their presence. Inhibition of methemoglobin reductase, considered in vivo as a possible mechanism of cobalt-mediated antagonism (60), is clearly not applicable in vitro and is unnecessary for effectiveness. No significant  $\text{SCN}^-$  was formed with  $\text{CoCl}_2$ , indicating that cobalt(II)-enhanced sulfurtransferase activity (e.g., possibly through increases in glutathione levels) (61) is not a factor in antagonism. It seems highly probable, then, that effectiveness of the antidote in the coincubation system is due to direct complexation of Co ion with cyanide in the medium, as has been proposed to occur in vivo (13-15). Since Co(II) forms a hexacyano complex with  $\text{CN}^-$  (13), when the KCN concentration in the system is 1.0 mM, 0.25 mM  $\text{CoCl}_2$  should reverse cyanide-induced ATP depression, whereas 0.10 mM should not. This is indeed what was observed (Table 2).

With thiosulfate, reversal of ATP depression in the coincubation system occurred concurrently with conversion of  $\text{CN}^-$  to  $\text{SCN}^-$ ; cyanmethemoglobin was not appreciably formed. Reversal of ATP by thiosulfate and  $\text{SCN}^-$  formation occurred with hepatocytes alone in the system and even when albumin was omitted from the medium. Presumably liver rhodanese, an enzyme with high substrate specificity and activity with thiosulfate, is responsible for the sulfurtransferase activity. Several investigators have cautioned against this interpretation outright, primarily based on experimental evidence showing that thiosulfate does not readily penetrate cell membranes and is not readily accessible to rhodanese located in the mitochondrial matrix (9,62). Furthermore, sulfane sulfur metabolism is complex and other enzymes may also be involved, making it exceedingly difficult to resolve the precise mechanism (46). Whatever the enzyme or enzymes involved and the mechanism of sulfur transfer, it is clear from the present data that intact hepatocytes do have the capability for directly utilizing thiosulfate for sulfane sulfur transfer to cyanide.

An unexpected and interesting outcome of these studies was that erythrocytes significantly increased the rate of antagonism of ATP depression by cyanide with thiosulfate antidote. Occasional reports of a rhodanese-like sulfurtransferase activity in erythrocytes have appeared in the literature (9,47,63), but the activity assayed as rhodanese was noted

to be very small and uncharacteristically labile (64). Whatever the identity of the active component, erythrocytes in our system also exhibited sulfurtransferase activity with thiosulfate in the absence of hepatocytes, confirming these earlier reports.

In general, these results are highly encouraging in demonstrating practical applications for the system. Some points still need to be addressed experimentally for a full understanding of the strengths and limitations of the approach. Specifically, (1) we need to demonstrate that cytochrome oxidase is in fact inhibited in the hepatocytes under the same experimental conditions in which ATP depression occurs. (2) We need to provide a satisfactory rationale for why higher cyanide concentrations in the medium are required for sensitive measurement of ATP depression compared with circulating levels in the bloodstream at death of the organism (65, 66) and for the sluggishness of ATP as an indicator in response to antidotal action; that is, are these factors relevant to the in vivo situation and, if not, what improvements (alternative indicators, alternative cell systems) can be recommended? (3) We need to obtain a better understanding of the nature of the sulfurtransferase activity in erythrocytes and its significance in cyanide detoxication with thiosulfate. (4) We need to document and compare antidotal effectiveness in cells from other species in addition to rats. (5) We need to initiate screening of other demonstrated antidotes against cyanide action to see whether they are effective in the system. These studies will be undertaken in the third year under the contract.

#### PLANNED STUDIES--THIRD YEAR

During the third year of the contract, research will continue on the mechanism of action of cyanide and various antidotes at the cellular level. Attempts will be made to identify the enzyme system in erythrocytes contributing to the effectiveness of sodium thiosulfate in reversing cyanide toxicity to hepatocytes in the in vitro system. Experiments will be continued with hepatocytes from other species, as available, for study of interspecies differences in response to cyanide and its antidotes.

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APPENDIX

Table A-1  
CONCENTRATION- AND TIME-DEPENDENT DEPRESSION OF HEPATOCYTE ATP LEVELS BY KCN<sup>a</sup>

KCN (mM)	0 Min <sup>b</sup>		Time After Cyanide Addition		5 Min <sup>b</sup>		10 Min <sup>b</sup>		30 Min <sup>b</sup>		120 Min <sup>b</sup>	
	ATP (10 <sup>-5</sup> M)	% of Control	ATP (10 <sup>-5</sup> M)	% of Control	ATP (10 <sup>-5</sup> M)	% of Control	ATP (10 <sup>-5</sup> M)	% of Control	ATP (10 <sup>-5</sup> M)	% of Control	ATP (10 <sup>-5</sup> M)	% of Control
0.0	1.35 ± 0.24		1.24 ± 0.32		1.16 ± 0.22		1.09 ± 0.39	0.92 ± 0.31				
0.10	1.25 ± 0.10	93.8 ± 10.2	1.04 ± 0.13	88.1 ± 24.0	0.97 ± 0.18	85.6 ± 0.9	0.74 ± 0.18	70.2 ± 11.2	0.73 ± 0.17	81.3 ± 10.8	0.71 ± 0.22	84.4 ± 11.0
0.25	1.27 ± 0.22	94.1 ± 2.7	0.85 ± 0.08	70.7 ± 10.3	0.79 ± 0.17 <sup>c</sup>	68.3 ± 5.4	0.50 ± 0.16 <sup>c</sup>	46.5 ± 3.0	0.55 ± 0.12 <sup>c</sup>	62.1 ± 11.6	0.52 ± 0.15	66.6 ± 6.5
0.50	1.16 ± 0.20	86.4 ± 3.7	0.75 ± 0.08 <sup>c</sup>	62.5 ± 10.3	0.65 ± 0.17 <sup>c</sup>	55.8 ± 6.6	0.31 ± 0.10 <sup>c</sup>	34.6 ± 5.9	0.35 ± 0.08 <sup>c</sup>	40.2 ± 8.1	0.26 ± 0.16 <sup>c</sup>	31.5 ± 13.9
1.0	1.02 ± 0.11	76.7 ± 6.3	0.80 ± 0.12 <sup>c</sup>	66.4 ± 10.4	0.57 ± 0.05 <sup>c</sup>	49.7 ± 1.7	0.31 ± 0.09 <sup>c</sup>	28.7 ± 4.4	0.26 ± 0.08 <sup>c</sup>	28.5 ± 4.5	0.07 ± 0.05 <sup>c</sup>	8.6 ± 5.0
2.5	1.14 ± 0.15	85.6 ± 8.0	0.81 ± 0.12 <sup>c</sup>	67.1 ± 9.3	0.55 ± 0.09 <sup>c</sup>	47.8 ± 4.7	0.24 ± 0.06 <sup>c</sup>	30.0 ± 12.5	0.19 ± 0.07 <sup>c</sup>	21.8 ± 6.2	0.11 ± 0.16 <sup>c</sup>	11.8 ± 15.7

<sup>a</sup> Rat hepatocytes were isolated by biopsy perfusion and cultured on collagen-coated plastic Petri dishes (1.5 × 10<sup>6</sup> attached cells/dish) for 24 hr in 4.0 ml of Waymouth's 752/1 medium supplemented with 0.1% albumin and hormones (21). To initiate the reaction, the culture medium was replaced by fresh medium containing the indicated concentration of cyanide and the dishes transferred to air-tight flasks and incubated at 37°C with gentle shaking. Separate flasks (in duplicate) were used for each sampling time.

<sup>b</sup> For 0, 2, and 5 min n = 3 experiments; for 10, 30, and 120 min n = 5 experiments except at 2.5 mM (n = 4). Data are means ± SD.

<sup>c</sup> Significantly different from controls (no KCN) at p < 0.05.

# CONCENTRATION- AND TIME-DEPENDENT FORMATION OF UREA BY HEPATOCYTES<sup>a</sup>

a Experimental protocol as in Table A-1. Data are means  $\pm$  SD for  $n = 5$  experiments except at 2.5 mM ( $n = 4$ ).

b Significantly different from controls (no KCN) at  $p < 0.05$ .

Table A-3

CONCENTRATION- AND TIME-DEPENDENT RELEASE OF LDH FROM HEPATOCYTES<sup>a</sup>

KCN (mM)	Time After Cyanide Addition					
	10 Min		30 Min		120 Min	
	LDH in Medium (U/L)	Net % <sup>b</sup> Release	LDH in Medium (U/L)	Net % Release	LDH in Medium (U/L)	Net % Release
0.0	39 ± 20(8)		51 ± 26(8)		70 ± 22(8)	
0.10	54 ± 28(5)	0.9 ± 0.9	67 ± 22(5)	0.7 ± 0.9	83 ± 19(5)	1.2 ± 1.3
0.25	49 ± 31(5)	0.7 ± 0.6	81 ± 24(5)	1.7 ± 0.8	109 ± 33(5) <sup>c</sup>	3.1 ± 2.3
0.50	58 ± 23(8) <sup>c</sup>	1.5 ± 0.6	100 ± 32(8) <sup>c</sup>	3.8 ± 1.9	157 ± 67(8) <sup>c</sup>	6.8 ± 3.9
1.0	57 ± 23(8)	1.4 ± 0.8	108 ± 31(8) <sup>c</sup>	4.7 ± 2.9	221 ± 113(8) <sup>c</sup>	11.6 ± 7.3
2.5	56 ± 18(7) <sup>c</sup>	1.8 ± 0.6	107 ± 33(7) <sup>c</sup>	5.0 ± 2.3	327 ± 160(7) <sup>c</sup>	19.5 ± 8.6
5.0	58 ± 20(4)	2.5 ± 2.8	110 ± 42(4) <sup>c</sup>	6.3 ± 2.9	385 ± 200(4) <sup>c</sup>	28.7 ± 19.3
						1166 ± 542(4) <sup>c</sup>
						84.3 ± 44.8

<sup>a</sup> Experimental protocol as in Table A-1. Data are means ± SD for number of experiments in parentheses.

<sup>b</sup> Net % release is release due to KCN after subtracting background control (no KCN) levels in medium. Mean total LDH available for release for the eight experiments reported was 1510 ± 184 U/L.

<sup>c</sup> Significantly different from controls (no KCN) at  $p < 0.05$ .



Table A-4

CAPABILITY OF SODIUM NITRITE AND SODIUM THIOSULFATE  
TO RESTORE ATP LEVELS IN COMBINED HEPATOCYTE/ERYTHROCYTE SYSTEM<sup>a</sup>

KCN (mM)	Antidote (mM)	ATP Levels <sup>b</sup> ( $10^{-5}$ M) at:			
		10 Min	30 Min	60 Min	120 Min
0.0	None	0.97 $\pm$ 0.50	0.90 $\pm$ 0.40	0.90 $\pm$ 0.44	0.85 $\pm$ 0.41
	NaNO <sub>2</sub> 5.0	1.02 $\pm$ 0.54	0.92 $\pm$ 0.48	0.95 $\pm$ 0.45	0.90 $\pm$ 0.39
	Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 10.0	1.04 $\pm$ 0.58	0.94 $\pm$ 0.41	0.84 $\pm$ 0.35	0.81 $\pm$ 0.38
	NaNO <sub>2</sub> + Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 5.0 10.0	0.95 $\pm$ 0.45	0.95 $\pm$ 0.47	0.78 $\pm$ 0.39	0.78 $\pm$ 0.32
1.0	None	0.38 $\pm$ 0.11	0.33 $\pm$ 0.06	0.25 $\pm$ 0.08	0.11 $\pm$ 0.05
	NaNO <sub>2</sub> 5.0	0.43 $\pm$ 0.09	0.46 $\pm$ 0.12	0.66 $\pm$ 0.32	0.49 $\pm$ 0.14
	Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 10.0	0.35 $\pm$ 0.13	0.44 $\pm$ 0.06	0.64 $\pm$ 0.38	0.74 $\pm$ 0.40
	NaNO <sub>2</sub> + Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 5.0 10.0	0.42 $\pm$ 0.17	0.65 $\pm$ 0.30	0.74 $\pm$ 0.39	0.79 $\pm$ 0.33

<sup>a</sup> Rat hepatocytes were isolated by biopsy perfusion and cultured on collagen-coated plastic petri dishes for 24 hr in 4.0 ml of Waymouth's 752 \* medium supplemented with 0.2% albumin and hormones ( $1.5 \times 10^6$  attached cells/dish). To start the reaction, the medium was replaced by aspiration with fresh medium containing erythrocytes ( $3 \times 10^9$  cells total; 1.6 g hemoglobin equivalents/dL) and KCN and the dishes were transferred to air-tight flasks. After a 10-min incubation in a water bath at 37°C with gentle shaking, the antidote was added quickly through the inlet and the reaction was continued for 120 min. Separate flasks (in duplicate) were used for each sampling time.

<sup>b</sup> In hepatocytes after removal of erythrocytes from reaction flasks by aspiration. Time is after antidote addition. Data are means  $\pm$  SD for n = 3 experiments.

Table A-5

RECOVERY OF ATP LEVELS AT 1.0 mM KCN WITH SODIUM NITRITE AND  
SODIUM THIOSULFATE IN COMBINED HEPATOCYTE/ERYTHROCYTE SYSTEM<sup>a</sup>

Antidote (mM)	ATP Levels <sup>b</sup> (%) at:			
	10 Min	30 Min	60 Min	120 Min
None	42 ± 11	39 ± 9	30 ± 7	14 ± 5
NaNO <sub>2</sub> 5.0	48 ± 16	55 ± 14	69 ± 8	57 ± 16
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 10.0	37 ± 8	45 ± 7	75 ± 13	91 ± 7
NaNO <sub>2</sub> + Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 5.0 10.0	45 ± 5	69 ± 4	94 ± 13	100 ± 13

<sup>a</sup> Experimental protocols and sampling times are as in Table A-4.  
Erythrocyte level was 1.6 g hemoglobin equivalents/dL.

<sup>b</sup> Percent of control (no KCN) levels in hepatocytes after removal of  
erythrocytes from reaction flasks by aspiration. Data are means ±  
SD for n = 3 experiments.

Table A-6

METHEMOGLOBIN FORMATION IN THE ABSENCE OF KCN  
BY SODIUM NITRITE IN COMBINED HEPATOCYTE/ERYTHROCYTE SYSTEM<sup>a</sup>

Antidote (mM)	Methemoglobin <sup>b</sup> (%) at:			
	10 Min	30 Min	60 Min	120 Min
None	0.1 ( 0.0, 0.2)	0.0 ( 0.0, 0.0)	0.4 ( 0.0, 0.8)	0.3 (0.0, 0.6)
NaNO <sub>2</sub> 5.0	31.5 (29.2, 33.8)	53.7 (55.6, 51.8)	68.7 (66.6, 70.7)	77.6 (72.9, 82.3)

<sup>a</sup> Experimental protocols are as in Table A-- except for omission of KCN. Erythrocyte level was 1.6 g hemoglobin equivalents/dL.

<sup>b</sup> Data are the means of n = 2 experiments, with individual values in parentheses.

Table A-7

CAPABILITY OF DMAP TO RESTORE ATP LEVELS  
IN COMBINED HEPATOCYTE/ERYTHROCYTE SYSTEM<sup>a</sup>

KCN (mM)	Antidote (mM)	ATP Levels <sup>b</sup> ( $10^{-5}$ M) at:			
		3 Min	10 Min	30 Min	120 Min
0.0	None	0.65	0.66 (0.69, 0.62)	0.56 (0.53, 0.58)	0.54 (0.53, 0.54)
	DMAP 0.25	0.74	0.61	0.73	0.49
	DMAP 0.50	0.66	0.58 (0.62, 0.54)	0.53 (0.49, 0.57)	0.43 (0.41, 0.44)
1.0	None	0.25	0.32 (0.30, 0.33)	0.29 (0.30, 0.27)	0.11 (0.13, 0.08)
	DMAP 0.25	0.36	0.48	0.52	0.47
	DMAP 0.50	0.32	0.45 (0.44, 0.45)	0.51 (0.50, 0.51)	0.36 (0.37, 0.34)

<sup>a</sup> Experimental protocols and sampling times are as in Table A-4.  
Erythrocyte level was 1.6 g hemoglobin equivalents/dL.

<sup>b</sup> In hepatocytes after removal of erythrocytes from reaction flasks by aspiration. Data are the means of  $n = 2$  experiments where individual values are given in parentheses; otherwise  $n = 1$ .

Table A-8

RECOVERY OF ATP LEVELS AT 1.0 mM KCN WITH DMAP IN  
COMBINED HEPATOCYTE/ERYTHROCYTE SYSTEM<sup>a</sup>

KCN (mM)	Antidote (mM)	ATP Levels <sup>b</sup> (%) at:			
		3 Min	10 Min	30 Min	120 Min
1.0	None	38	48 (43,53)	52 (57,47)	20 (25,15)
	DMAP 0.25	49	79	71	96
	DMAP 0.50	48	72 (71,73)	95 (102,88)	77 (90,63)

<sup>a</sup> Experimental protocols and sampling times are as in Table A-4.  
Erythrocyte level was 1.6 g nemoglobin equivalents/dL.

<sup>b</sup> Percent of control (no KCN) levels in hepatocytes after removal of  
erythrocytes from reaction flasks by aspiration (data in Table A-7).  
Data are means of n = 2 experiments, where individual values are given  
in parentheses; otherwise n = 1.

Table A-9

CYTOTOXICITY OF COBALT COMPOUNDS IN  
THE COMBINED HEPATOCYTE/ERYTHROCYTE SYSTEM<sup>a</sup>

Antidote (mM)		LDH Release (%) <sup>b</sup> at:		
		10 Min	30 Min	120 Min
Cobalt(II) bis-bipyridyl	0.25	0.07	0.0	0.0
	0.50	0.10	0.0	1.43
	1.0	0.15	0.0	0.40
	5.0	0.0	0.0	1.60
Cobalt(II) chloride	0.25	0.0	0.0	0.15
	0.50	0.0	0.12	0.75
	1.0	0.20	0.30	0.0
	5.0	0.0	0.05	0.0
Hydroxy- cobalamin	0.25	0.96	3.01	0.15
	0.50	0.91	2.24	0.03
	1.0	1.25	-- <sup>b</sup>	0.0
	5.0	2.33	-- <sup>b</sup>	1.65

<sup>a</sup> Release from the total system after subtracting background (control cell) LDH release. Total LDH activity available = 4104 U/L (2805 U/L for erythrocytes + 1299 U/L for hepatocytes). Experimental protocols are as in Table A-4 except that (1) LDH was measured in the medium of the same flasks by serial sampling, and (2) cells were actually exposed for noted times plus 10 min to simulate exposure time in antidote study where they were added 10 min prior to KCN.

<sup>b</sup> Each data point is the mean of two replicates.

<sup>c</sup> These assays were not conducted due to insufficient number of erythrocytes for the experiment.

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